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Protein turnover as a component in the light/dark regulation of phosphoenolpyruvate carboxylase protein-serine kinase activity in C₄ plants

(regulatory phosphorylation/C₄ photosynthesis/light activation/protein kinase/cycloheximide)

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ABSTRACT Maize leaf phosphoenolpyruvate carboxylase [PEPC; orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] protein-serine kinase (PEPC-PK) phosphorylates serine-15 of its target enzyme, thus leading to an increase in catalytic activity and a concomitant decrease in malate sensitivity of this cytoplasmic C₄ photosynthesis enzyme in the light. We have recently demonstrated that the PEPC-PK activity in maize leaves is slowly, but strikingly, increased in the light and decreased in darkness. In this report, we provide evidence that cycloheximide, an inhibitor of cytoplasmic protein synthesis, when fed to detached leaves of C₄ monocots (maize, sorghum) and dicots (*Portulaca oleracea*) in the dark or light, completely prevents the *in vivo* light activation of PEPC-PK activity regardless of whether the protein kinase activity is assessed *in vivo* or *in vitro*. In contrast, chloramphenicol, an inhibitor of protein synthesis in chloroplasts, has no effect on the light activation of maize PEPC-PK. Similarly, treatment with cycloheximide did not influence the light activation of other photosynthesis-related enzymes in maize, including cytoplasmic sucrose-phosphate synthase and chloroplast stromal NADPH-malate dehydrogenase and pyruvate, P_i dikinase. These and related results, in which detached maize leaves were treated simultaneously with cycloheximide and microcystin-LR, a potent *in vivo* and *in vitro* inhibitor of the PEPC type 2A protein phosphatase, indicate that short-term protein turnover of the PEPC-PK itself or some other essential component(s) (e.g., a putative protein that modifies this kinase activity) is one of the primary levels in the complex and unique regulatory cascade effecting the reversible light activation/seryl phosphorylation of PEPC in the mesophyll cytoplasm of C₄ plants.

Light reversibly activates a number of photosynthesis-related enzymes in plants via several different mechanisms (1–4). Among these is the light activation of leaf cytoplasmic phosphoenolpyruvate carboxylase [PEPC; orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31] in C₄ plants by reversible protein phosphorylation (5, 6). Previous *in vitro* (7, 8) and *in vivo* (9) studies with maize leaf PEPC demonstrated that the phosphorylation of a single, N-terminal seryl residue (Ser-15) leads to an increase in catalytic activity and a decrease in feedback inhibition of the target enzyme by L-malate. Related findings from a reconstituted phosphorylation system indicated that the activity of the protein-serine kinase that catalyzes this regulatory phosphorylation of PEPC is not affected by a number of putative, light-modulated cytoplasmic effectors (e.g., reduced thioredoxin *h*, Ca²⁺, PP_i, fructose 2,6-bisphosphate) and autophos-

phorylation (6, 7). However, more recent work has established that the phosphoenolpyruvate carboxylase protein-serine kinase (PEPC-PK) is activated by light and inactivated by darkness *in vivo* (10). Moreover, this striking regulatory process appears independent of SH status, Ca²⁺ levels, and a putative, tight-binding PEPC-PK effector (10).

One of the distinguishing features of the reversible light activation of PEPC-PK and its target enzyme, PEPC, in C₄ plants is its sluggishness *in vivo*; when compared to the *in vivo* activation of photoregulated mesophyll chloroplast stromal enzymes such as pyruvate, P_i dikinase (PPDK) and NADPH-malate dehydrogenase (MDH) (2, 6), the former are both relatively slow processes, taking up to 1 hr, rather than minutes, for completion (10–12). To gain more insight into this difference and the specific mechanism(s) by which the PEPC-PK activity *in vivo* is slowly, but strikingly, increased in the light and decreased in darkness (10), detached maize leaves were fed two widely used inhibitors of protein synthesis. PEPC-PK activity was subsequently assessed either *in vivo* [malate IC₅₀ values for inhibition of the target enzyme (11, 12)] or *in vitro* [³²P phosphorylation of purified dark-form PEPC (7, 10)]. Whereas chloramphenicol (CAP), a 70S ribosome-specific inhibitor of chloroplastic protein synthesis, had no effect on the light activation of PEPC-PK, cycloheximide (CHX), an inhibitor of cytoplasmic protein synthesis, completely blocked the light activation of this protein-serine kinase. In contrast, the *in vivo* activation of several other photoregulated cytoplasmic [sucrose-phosphate synthase (SPS)] and chloroplastic (PPDK, MDH) photosynthesis-related enzymes was not influenced by CHX treatment. These results indicate that the synthesis and degradation of PEPC-PK *per se* or some other essential component(s) are involved at one of the primary levels in the regulatory cascade effecting the reversible light activation/seryl phosphorylation of PEPC in the mesophyll cytoplasm of C₄ plants.

MATERIALS AND METHODS

Materials. Maize (*Zea mays* L., cv. Golden Cross Bantam) plants were grown as described (7, 10). [γ-³²P]ATP [specific activity, 3000 Ci (111 TBq)/mmol] was purchased from Amersham. Dark-form maize leaf PEPC was purified by the procedure described (7, 8). All biochemical reagents were obtained from Sigma except for microcystin-LR (MC) (Cal-

Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEPC-PK, phosphoenolpyruvate carboxylase protein-serine kinase; SPS, sucrose-phosphate synthase; PPDK, pyruvate, P_i dikinase; MDH, NADPH-malate dehydrogenase; CHX, cycloheximide; CAP, chloramphenicol; MC, microcystin-LR.

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biochem). Stock solutions of 10 mM CHX and 310 mM CAP were prepared in absolute ethanol, while 0.5 mM MC was dissolved in 20% (vol/vol) methanol.

Feeding of Protein Synthesis and Protein Phosphatase Inhibitors. Preilluminated leaves (≈ 2 g fresh wt each) from 4- to 6-week-old maize plants were excised underwater, inserted into 150-ml beakers containing 100 ml of distilled water (control), 5 μ M CHX, 310 μ M CAP, or 10 nM MC in water and maintained at room temperature. When feeding was done in the dark, the beakers were placed in a darkened fume hood overnight. The dark sample was then prepared from these leaves and the corresponding light sample was collected after a 90-min illumination of the tissue. When feeding was done in the light, detached control leaves that had been preilluminated for 1.5 hr in water were either maintained in water or fed inhibitors for 4 hr in continued light, followed by preparation of leaf extracts. Illumination was provided by a forced-air cooled 300-W, low-temperature lamp at an incident light intensity of 600–800 μ E \cdot m $^{-2}$ ·s $^{-1}$ (E, einstein) (400–700 nm).

Preparation of Leaf Extracts. Samples (0.3 g fresh wt) from the control or inhibitor-treated leaf material were chopped and ground at 4°C in a prechilled mortar containing washed sand, 2% (wt/vol) insoluble polyvinylpyrrolidone, and 1.5 ml of the appropriate extraction buffer. Buffer A [0.1 M Tris-HCl, pH 8.0/20% (vol/vol) glycerol/10 mM MgCl $_2$ /14 mM 2-mercaptoethanol/1 mM EDTA] was used for preparation of PEPC and its protein-serine kinase; buffer B (buffer A plus 2 mM pyruvate) was used for PPK; buffer C (50 mM Mops-NaOH, pH 7.5/15 mM MgCl $_2$ /2.5 mM dithiothreitol/1 mM EDTA/0.1% Triton X-100) was used for SPS; and buffer D (0.1 M Tris-HCl, pH 8.0/1 mM EDTA/14 mM 2-mercaptoethanol) was used for MDH. The crude leaf homogenates were filtered through an 80- μ m nylon net and centrifuged for 1.5 min at 8700 \times g. The supernatant fluid was either used immediately (PPDK, MDH) or after a 0.2-ml aliquot was rapidly desalted at 4°C on a Sephadex G-25 column (1 \times 5 cm) equilibrated with 0.1 M Tris-HCl, pH 7.5/10 mM MgCl $_2$ /20% (vol/vol) glycerol for PEPC and PEPC-PK or buffer C minus Triton X-100 for SPS.

Activity Assays. PEPC activity was determined spectrophotometrically at 340 nm and 30°C. The assay mixture (12) contained, in a total vol of 1 ml, 50 mM Hepes-KOH (pH 7.3), 2.5 mM phosphoenolpyruvate, 5 mM MgCl $_2$, 1 mM NaHCO $_3$, 0.2 mM NADH, 10 units of malate dehydrogenase, various concentrations of L-malate, and 10 μ l of desalted extract (added last). Malate IC $_{50}$ values were taken as the malate concentration required for 50% inhibition of PEPC activity under these assay conditions. PEPC-PK activity was measured by 32 P incorporation from [γ - 32 P]ATP into purified dark-form PEPC (10). The phosphorylation mixture contained 35 μ l of desalted extract, 10 μ g of purified dark-form maize PEPC, an adenylate kinase inhibitor plus a creatine kinase/phosphocreatine ADP-scavenging system (10), 25 μ M ATP, and 3 μ Ci of [γ - 32 P]ATP in a final vol of 60 μ l. After 45 min of incubation at 30°C, the reaction was stopped by adding 20 μ l of SDS sample buffer (0.25 M Tris-HCl, pH 6.8/8% SDS/40% glycerol/20% 2-mercaptoethanol), followed by immediate boiling for 2 min. Vertical SDS/PAGE was performed as described (13, 14), and autoradiographs were prepared from the dried gels with Kodak X-Omat AR film and two Lightning Plus intensifying screens (DuPont) at -80°C.

SPS, PPK, and MDH activities were measured according to ref. 15 (at limiting substrate concentrations plus 10 mM P $_i$ at 25°C), ref. 16 (forward direction plus 2.5 mM glucose 6-phosphate and 2 units of purified maize PEPC at 30°C), and ref. 17 at 30°C, respectively.

RESULTS AND DISCUSSION

Effects of CHX, CAP, and MC on the Light-Induced Changes in Malate Sensitivity of Maize Leaf PEPC. The IC $_{50}$ values for PEPC inhibition by L-malate were used as an indirect means of following the effect of dark to light transitions on the apparent *in vivo* activity of the PEPC-PK since these values reflect the seryl-phosphorylation status of the target enzyme both *in vitro* (7, 8) and *in vivo* in response to light and dark (9–12). Feeding 5 μ M CHX to detached preilluminated maize leaves in the dark overnight completely and reproducibly prevented the subsequent light-induced increase in the malate IC $_{50}$ value of PEPC without having any significant effect on the dark-form enzyme (Table 1). In contrast, CAP treatment had no effect on the light-induced changes in malate sensitivity of PEPC (Table 1). Overnight feeding of 5 μ M CHX in the dark to predarkened maize leaves had the same inhibitory effect on light activation of PEPC. Results similar to those presented in Table 1 were obtained when detached leaves of sorghum, another C $_4$ grass, and halved leaves of *Portulaca oleracea*, a C $_4$ dicot, were fed CHX (data not shown).

Given that such inhibitors are known not to be absolutely specific, thus possibly causing detrimental side effects (18), and that the 3-(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive light activation/phosphorylation of PEPC occurs in the cytoplasm and is somehow related to photosynthetic electron transport and/or photophosphorylation (5, 6, 19), it was imperative to examine the effect of CHX treatment on the *in vivo* light activation of other photosynthesis-related enzymes in maize. Cytoplasmic SPS and chloroplast stromal PPK are, like PEPC, light-activated by reversible phosphorylation/dephosphorylation cycles (2, 3, 6, 15, 20). In contrast, stromal MDH is light activated by 3-(3,4-dichlorophenyl)-1,1-dimethylurea-sensitive changes in its SH redox status mediated by noncyclic electron flow and the chloroplastic ferredoxin/thioredoxin *m* system (1, 2, 17, 21). Notably, the results (Table 2) indicate that the light activation of these three enzymes was not significantly affected by feeding 5 μ M CHX to detached maize leaves under conditions identical to those described in Table 1. Similarly, CHX treatment of detached leaves had no obvious effect on either their total soluble protein content (mg/g fresh wt) or polypeptide pattern (e.g., see Fig. 1A, lane 2 versus 3 and lane 5 versus 6) over the duration of these relatively short-term experiments. Thus, the inhibitory effect of CHX on the apparent *in vivo* activity of the PEPC-PK (Table 1) appears rather selective for the light activation of this specific converter enzyme.

From the results described above, it is clear that *de novo* synthesis of PEPC-PK or some other essential component(s) (e.g., a putative modifying protein that activates this protein-serine kinase *in vivo*) is induced during a 1.5-hr exposure to light. Thus, it was anticipated that if CHX were fed to illuminated detached leaf tissue after a point at which sufficient protein (i.e., either PEPC-PK or the putative modifying

Table 1. Effects of protein synthesis inhibitors on light-induced increase in the malate IC $_{50}$ value of maize leaf PEPC

Inhibitor	Malate IC $_{50}$, mM		
	Light (L)	Dark (D)	L/D
None (control)	0.45	0.18	2.5
CHX	0.20	0.17	1.2
CAP	0.46	0.18	2.6

Preilluminated maize leaves were excised and fed water (control), 5 μ M CHX, or 310 μ M CAP in the dark overnight. Dark-form PEPC extracts were then prepared from these leaves and the corresponding light-form extracts were made after a 90-min illumination of the tissue.

Table 2. Effects of CHX treatment on *in vivo* light activation of SPS, PPK, and MDH in detached maize leaves

Enzyme	Light/dark activity ratio	
	Control*	+ 5 μ M CHX
SPS†	6.5	5.5
PPDK	7.0	6.9
MDH	11.7	12.7

See Table 1 and *Materials and Methods* for experimental details.

*The light-activated activities (in μ mol per min per mg of soluble protein) of SPS, PPK, and MDH were 0.14, 0.43, and 0.16, respectively.

†Activity was determined at limiting substrate concentrations in the presence of the inhibitor P_i (15).

protein) had already been synthesized in the light so that PEPC was phosphorylated and in its high malate IC₅₀ form, continued illumination of the inhibitor-fed tissue would maintain the target enzyme in its high activation (phosphorylation) state. This reasoning assumes that the activity of the PEPC type 2A protein phosphatase (22) is relatively low in the light and/or that rapid degradation of the newly synthesized PEPC-PK or putative modifying protein does not take place in the light. However, when preilluminated (1.5 hr in the light) control leaf tissue was placed in water or 5 μ M CHX and maintained in the light for an additional 4 hr, the malate IC₅₀ value remained constant in the absence of the inhibitor but decreased to a level characteristic of dark-form PEPC in the presence of CHX (Table 3). These data suggest that (i) the PEPC type 2A protein phosphatase is active in the light, and (ii) there is net turnover of the PEPC-PK or putative modifying protein in the light in the absence of its synthesis, thereby leading to a net dephosphorylation of PEPC and the resulting decrease in its malate IC₅₀ value. Based on these observations and suggestions, a more critical and revealing experiment was designed. Control detached leaves that had been preilluminated for 1.5 hr were fed 5 μ M CHX and 10 nM MC, alone and in combination, for 4 hr in the light. This latter cyclic heptapeptide is a potent and specific inhibitor of plant and animal type 1 and 2A protein phosphatases both *in vitro* and *in vivo* (23–25). In the absence of the protein synthesis inhibitor, both the control and MC-treated illuminated tissue maintained PEPC in its high malate IC₅₀ form (Table 3). In contrast, in the presence of CHX alone, the activation (phosphorylation) state of the target enzyme collapsed back to a malate IC₅₀ value characteristic of the dark-form enzyme (see above and Table 3). However, in the presence of both inhibitors, the CHX-induced decrease in the malate IC₅₀ value was largely prevented, clearly indicating that the protein phosphatase that dephosphorylates light-form PEPC *in vivo* remains totally active in the light and is of the type 1 or type 2A class (23–25), as previously implicated by *in vitro* studies (22). Thus, the regulatory phosphorylation status of PEPC is mainly determined by its light-activated protein-serine kinase (10), the latter of which appears to be light/dark

Table 3. Effects of protein synthesis and protein phosphatase inhibitors on the maintenance of the high malate IC₅₀ form of PEPC in the light

Inhibitor(s)	Malate IC ₅₀ , mM
None (control)	0.46
CHX	0.17*
MC	0.42
CHX + MC	0.35

Detached control leaves, preilluminated for 1.5 hr in water, were subsequently maintained in water (control) or fed 5 μ M CHX, 10 nM MC, or both for an additional 4 hr in the light before extraction and assay of PEPC activity with or without L-malate.

*Dark-form PEPC has a malate IC₅₀ value of \approx 0.2 mM (see Table 1).

modulated by the relative rates of its synthesis and degradation in the cytoplasm of both C₄ monocots (maize, sorghum) and dicots (*P. oleracea*).

Effects of Protein Synthesis Inhibitors on PEPC-PK Activity.

One question arising from the malate-sensitivity experiments described above (Tables 1 and 3) is whether CHX inhibits the apparent *in vivo* PEPC-PK activity by direct interaction with this converter enzyme, a putative modifying protein, or PEPC *per se*. To address this and other issues, *in vitro* ³²P phosphorylation assays of PEPC-PK activity were performed with rapidly prepared, desalted leaf extracts and purified dark-form PEPC as the protein substrate (10). Fig. 1 shows the effect of CHX and CAP on light activation of PEPC-PK activity when the inhibitors were fed to preilluminated detached maize leaves in darkness overnight. While the activity of the PEPC-PK is low in darkness and high in the light for the control (10) and CAP-treated tissue (cf. lane 2 versus 5 and lane 4 versus 7, respectively, in Fig. 1B), feeding 5 μ M CHX completely prevented this striking light activation of the protein-serine kinase (cf. lane 3 versus 6 in Fig. 1B). When either CHX (5 μ M) or CAP (310 μ M) was added directly to the phosphorylation assay mixture containing the desalted extract from the illuminated control leaf tissue, no *in vitro* inhibition of PEPC-PK activity was observed (cf. lanes 5, 8, and 9 in Fig. 1B). Similarly, when detached control leaves, preilluminated for 1.5 hr in water, were fed CHX (5 μ M) for an additional 4 hr in the light, PEPC-PK activity was totally inhibited (cf. lanes 2 and 4 versus lane 3 in Fig. 2B). The presence of both CHX and MC also led to a complete inhibition of PEPC-PK activity (Fig. 2B, lane 5), even though the malate IC₅₀ value of the endogenous PEPC was relatively

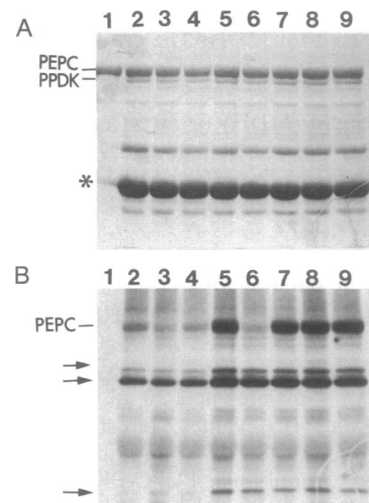


FIG. 1. Light activation of PEPC-PK activity and the effects of protein synthesis inhibitors *in vivo* and *in vitro*. Preilluminated maize leaves were detached and fed water, 5 μ M CHX, or 310 μ M CAP in the dark overnight. The dark-form PEPC-PK was extracted before illumination of these leaves and the corresponding light-form protein kinase was extracted after a 90-min illumination (cf. Table 1). PEPC-PK activity in desalted leaf extracts was determined by ³²P phosphorylation of purified dark-form PEPC as described in *Materials and Methods* and in ref. 10. (A) SDS gel stained with Coomassie blue R-250. (B) Corresponding autoradiograph of A. Desalted extracts were prepared from darkened (lanes 2–4) and illuminated (lanes 5–9) leaf tissue fed water (lanes 2, 5, 8, and 9), 5 μ M CHX (lanes 3 and 6), or 310 μ M CAP (lanes 4 and 7). Lanes 8 and 9, experiments in which 5 μ M CHX and 310 μ M CAP, respectively, were added directly to the *in vitro* assay mixture of PEPC-PK activity extracted from illuminated control leaf tissue. Lane 1, purified dark-form PEPC. The 43-kDa creatine kinase monomer [* (see ref. 10)] and the 95-kDa PPK and 110-kDa PEPC subunits are indicated in A. Arrows in B point to other light-enhanced but CHX-insensitive phosphoproteins.

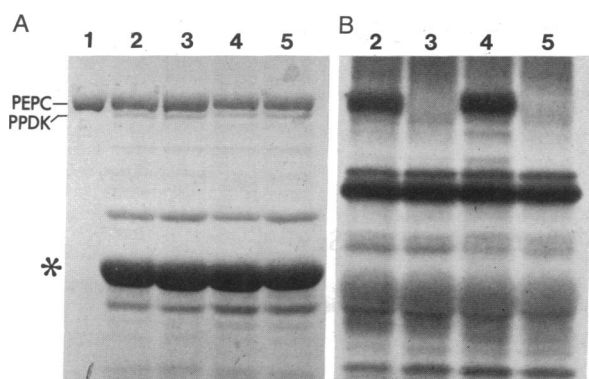


FIG. 2. Effects of CHX and MC on PEPC-PK activity when fed for 4 hr in the light to detached control leaves that had been preilluminated for 1.5 hr in water before treatment (cf. Table 3). PEPC-PK was assayed by ^{32}P phosphorylation of purified dark-form PEPC as described in *Materials and Methods* and in ref. 10. (A) SDS gel stained with Coomassie blue R-250. (B) Corresponding autoradiograph of A. Desalted extracts were prepared from preilluminated control leaf tissue that was maintained in water (lanes 2) or fed 5 μM CHX (lanes 3), 10 nM MC (lanes 4), or 5 μM CHX plus 10 nM MC (lanes 5) for an additional 4 hr in the light. Lane 1, purified dark-form PEPC. The creatine kinase (*), PPK, and PEPC monomers are indicated in A.

high under these conditions (Table 3). These collective findings further support the view that the PEPC type 2A protein phosphatase(s) (22) remains fully active in the light, even in the presence of CHX, and is effectively inhibited by nanomolar concentrations of MC *in vivo*. Except in the presence of both CHX and MC, whenever the *in vitro* PEPC-PK activity was low, so was the malate IC_{50} value of the endogenous PEPC from the corresponding leaf tissue and vice versa (cf. Table 1 versus Fig. 1B and Table 3 versus Fig. 2B).

It is notable that while the *in vivo* light activation of PEPC-PK activity is completely inhibited by feeding CHX to detached leaves (Fig. 1B), other soluble leaf proteins that are phosphorylated *in vitro* by endogenous protein kinases are not affected by such treatment; this includes even those polypeptides whose *in vitro* phosphorylation status is greater in the light than in the dark extracts (see arrows in Fig. 1B). Therefore, it is evident from the present study that the inhibitory effect of this cytoplasmic protein synthesis inhibitor is quite specific for the PEPC-PK or some other essential component(s) (e.g., its putative modifying protein), without having significant effects on other protein kinase and/or protein phosphatase activities (Fig. 1B) and light-activation systems (Table 2).

CONCLUDING REMARKS

The present study demonstrates that the *in vivo* light/dark regulation of PEPC-PK activity in C_4 leaves (10) involves net *de novo* cytoplasmic protein synthesis in the light and subsequent degradation in darkness. At present, it is not known what essential component(s)—e.g., PEPC-PK itself or a putative modifying protein—is the target of this unique light-modulation system. The increasingly complex PEPC regulatory cascade, involving at least protein synthesis/degradation and phosphorylation/dephosphorylation cycles, is totally different from other well-known mechanisms of reversible light activation of photosynthesis-related enzymes (1–4, 6, 15) and may well explain why the light/dark modulation of PEPC-PK (10) and its target enzyme PEPC (11, 12, 19) is so much slower than that of other photoactivated

enzymes (e.g., PPK, MDH). It is obvious that further purification of the PEPC-PK (7) and the subsequent production of monospecific antibodies against this protein will elucidate whether, indeed, this specific enzyme is the target of this unique, protein turnover-based regulatory system.

In addition, our findings raise several interesting questions as to how photosynthesis-related (5, 6, 19) light and dark signals so specifically influence cytoplasmic protein synthesis and degradation, respectively, and whether short-term protein turnover is, like other posttranslational covalent modifications, a general mechanism for regulating enzyme activity in plants in response to external and internal stimuli. Clearly, our results indicate that this is probably not the case with respect to the light-activation systems associated with other photosynthesis-related cytoplasmic and stromal enzymes.

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Biochemistry. In the article “Transforming function of proto-*ras* genes depends on heterologous promoters and is enhanced by specific point mutations” by Asit K. Chakraborty, Klaus Cichutek, and Peter H. Duesberg, which appeared in number 6, March 1991, of *Proc. Natl. Acad. Sci. USA* (88, 2217–2221), the authors wish to add the following to the Acknowledgments. “The generous advice and experimental help of Shelley Blam, Michael Pech, and C. Durago Rao to P.H.D. while on sabbatical in Stuart Aaronson’s lab at the National Cancer Institute are gratefully recorded.”

Botany. In the article “Protein turnover as a component in the light/dark regulation of phosphoenolpyruvate carboxylase protein-serine kinase activity in C₄ plants” by Jin-an Jiao, Cristina Echevarría, Jean Vidal, and Raymond Chollet, which appeared in number 7, April 1991, of *Proc. Natl. Acad. Sci. USA* (88, 2712–2715), the authors request that the following correction be noted. The concentration of microcystin-LR (MC) supplied to the detached maize leaves for 4 hr in the light was 10 μ M (not nM) (see Fig. 2, Table 3, and related text).